GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION
SPONSORED PROJECT INITIATION

Date: May 23, 1979

Project Title: Natural Anti-Tumor Agents from the Senecioneae

Project No: G-33-P02 Green card

Project Director: Dr. Leon H. Zalkow

Sponsor: DHEW/PHS/NIH - National Cancer Institute; Bethesda, MD 20014

Agreement Period: From 4/1/79 Until 3/31/80 (02 Year)

Type Agreement: Grant No. 5 R01 CA23277-02

Amount: $40,783 New PHS Funds (G-33-P02)
8,177 GIT Contribution (G-33-342)
$48,960 Total

Reports Required: Annual Progress Reports with Continuation Applications
Terminal Progress Report upon Grant Expiration

Sponsor Contact Person (s):

Technical Matters

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Division of Cancer Treatment
National Cancer Institute
Bethesda, MD 20014

Contractual Matters
(thru OCA)

Ms. Marian Focke
Grants Management Contact
National Cancer Institute
Bethesda, MD 20014

Phone: (301) 496-7444

NOTE: FOLLOW-ON PROJECT TO G-33-635.

Defense Priority Rating: None

Assigned to: Chemistry (School/Laboratory)

COPIES TO:

Project Director
Division Chief (EES)
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Project File (OCA)
Project Code (GTRI)
Other

CA-3 (3/76)

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION

Date: 5/23/80

Project Title: Natural Antitumor Agents from the Senecioneae

Project No: G-33-P02

Project Director: Dr. Leon H. Zalkow

Sponsor: DHEW/PHS/NIH - National Cancer Institute;
Bethesda, MD 20014

Effective Termination Date: 3/31/80 (02 year)

Clearance of Accounting Charges: --------

Grant/Contract Closeout Actions Remaining:

_ Final Invoice and Closing Documents
_ Final Fiscal Report
_ Final Report of Inventions
_ Govt. Property Inventory & Related Certificate
_ Classified Material Certificate
x Other Annual Report of Expenditures due by 6/30/80 (for 02 year)

NOTE: Continued by G-33-P03 (03 year)

Assigned to: Chemistry (School/~~Laboratory~~)

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Project Code (GTRI)
Other C. E. Smith

CA-4 (1.79)

SECTION IV

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1 →	GRANT NUMBER
SECTION IV—SUMMARY PROGRESS REPORT	CA 23277-03 G-33-P02 Interim Progress Report submitted 1/21/80 w/ proposal
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)	PERIOD COVERED BY THIS REPORT
Zalkow, Leon H.	FROM 1/1/79 THROUGH 1/1/80
NAME OF ORGANIZATION: Georgia Institute of Technology	

TITLE (Repeat title shown in Item 1 on first page)

Natural Antitumor Agents from the Senecioneae

1. List publications: (a) published and not previously reported; (b) in press. Provide five reprints if not previously submitted.
2. List all additions and deletions in professional personnel and any changes in effort.
3. Progress Report. (See Instructions)

1) L. Gelbaum, L. H. Zalkow, and S. Bonetti, "Pyrrolizidine Alkaloids from Senecio smallii, 20th Annual Meeting of the American Society of Pharmacognosy, Purdue University, July 20-Aug. 3, 1979.

L. H. Zalkow, L. T. Gelbaum, and D. Van Derveer, "Eremophilane Sesquiterpenes from Senec aureus," J. Chem. Soc., Perkin I, 1542 (1979).

L. H. Zalkow, S. Bonetti, L. Gelbaum, M. M. Gordon, B. B. Patil, A. Shani, and D. Van Derveer, "Pyrrolizidine Alkaloids from Middle Eastern Plants," J. Natural Products, in press.

2) During the period covered the following personnel devoted the indicate amounts of time.

Dr. L. Gelbaum, Research Scientist, 100%
Ms. S. Bonetti, B.S., 50%

3) During the period Jan. 1, 1979-Jan. 1, 1980 we have continued our work on the extracts of the Senecionaea and Crotaloria spectabilis. The following is a list of the Senecio species, collected in Colorado, that have been extracted with 95% ethanol and submitted for screening by the National Cancer Institute (NCI):

Senecio triangularis Hooker
S. streptanthifolius Greene
S. integerrimus Cronquist, Var. exaltatus (Nutall)
S. werneriaefolius A. Gray
S. fremontii Cronquist, Var. blitoides (Greene)
S. fendleri A. Gray
S. crassulus A. Gray
S. pseudaureus Greenman, Var. flavulus (Greene)
S. atratus Greene
S. dimorphophyllus Greene, Var. dimorphophyllus
S. eremophilus (Rydberg) Greenman. Var. kingii

Extracts from Crotalaria spectabilis Roth, Dicerandra linearfolia, Serenoa repens and Ophioglossium crotalophoroide have also been submitted for screening but results for the above plants have not yet been received.

NCI screening for ethyl-1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate (1) isolated from Senecio anonymus Wood (previously Senecio smallii Britt.) is now in progress. Preliminary KB screening results indicate significant activity with an ED_{50} of 3.3 x 10(0). The in vivo testing of 1 is now underway.

Using high performance liquid chromatography we have been able to isolate and identify a rare pyrrolizidine alkaloid neosenkirkine (2), and a new pyrrolizidine alkaloid which is the trans cinnamic acid ester of hydroxy senkirkine (3). These are in addition to senecionine (4), retrorsine (5), and senkirkine (6) previously isolated.

U.S. GOVERNMENT PRINTING OFFICE: 1978 260-999/3198

What is the mechanism of the antitumor effects of indicine N-oxide? The metabolism to indicine has clearly been shown not to be the mechanism (Powis, 1979). Many years ago it was suggested that pyrrolizidine alkaloids may interfere with purine metabolism in some unknown manner (Culvenor, 1968). It has been suggested that N-oxides act as biological oxygenating agents, particularly with respect to purines (Murray, 1976). It is known that the stacking properties of adenine nucleotides are absent in the corresponding nucleotide N-oxides (Mantsch, 1975). However, all of this is at present speculation and there is little evidence to suggest how indicine N-oxide might act as an antitumor agent.

Thus, with this proposal we hope to fill gaps in the knowledge of the mechanism of the antitumor effects of indicine N-oxide. Structure activity relationships will be developed by acylating retronecine[a] and thus discovering the critical feature necessary in the side chain of indicine N-oxide. Microbiological modifications of existing side chains will also be undertaken. We also plan to develop collagenase-sensitive pepidyl pyrrolizidines as potential latentiated antitumor agents (Marguisee, 1978).

C. Progress Report: 4/1/78 - 4/30/80

Dr. Leon H. Zalkow, Professor and Principal Investigator, 4/1/78-4/30/80, 25% time.

Dr. Leslie T. Gelbaum, Research Scientist and Co-Principal Investigator, 4/1/78-4/30/80, 100% time.

Dr. Howard Deutsch, Research Scientist, 4/1/78-1/1/79, 25% time.

Ms. S. Bonetti, B.S., Technician, 4/1/78-9/1/79, 50% time; graduate research assistant 9/1/79-4/30/80, 20% time.

Dr. Maureen M. Gordon, Postdoctoral fellow, 3/1/80-4/1/80, 50% time.

In the previous application we listed the following specific aims: (1) To fractionate and screen Senecio aureus and S. smallii; (2) To arrange for the collection of a number of species of Senecio and to process and screen as many of these as possible; (3) To attempt to shed some light on structure activity relationships by examining the NCI screening data on pyrrolizidine alkaloids and eremophilane sesquiterpenoids, if the latter exists; (4) To provide semi-synthetic pyrrolizidine alkaloid-like compounds for screening. We shall discuss each of these aims and our results.

Plant Extracts and Compounds Submitted for Screening to NCI During Grant Period 4/1/78 - 2/29/80[b]

Table 1 in Appendix A lists all plants, their extracts and the NCI assigned numbers submitted for screening during the period designated followed by copies of the actual screening data. The plants were collected and identified by four American and one Israeli botanist. The plants were processed according to the NCI procedure of 2/22/77. Table 1 lists sixty (60) plant extracts and fractions and the two pyrrolizidine alkaloid N-oxides, echinatine N-oxide and europine N-oxide. Of the plant extracts screened only the ethanol extract of Senecio smallii (Senecio anonymous Wood) (NSC B837136) showed good activity T/C 133, dose 174 mg/Kg) and this activity was concentrated in the aqueous methanol fraction (NSC B837138, T/C 137, dose 50 mg/Kg). We have isolated six compounds from this methanol fraction, five of which are the

b) This list also includes related pyrrolizidine alkaloid bearing plants from Israel obtained under grant CA-19946 (Carcinogenic Plants of the Dead Sea Area. 6/30/76-6/30/79). No attempt was made to renew grant CA-19946 because our interest shifted to anti-tumor activity and in-particular to the semi-synthetic work described in this proposal (CA-23277).

pyrrolizidine alkaloids[a]: senecionine (NSC 089935), retrorsine (NSC 107659), the seco-pyrrolizidine alkaloids senkirkine and neosenkirkine and the trans cinnamic acid ester of hydroxysenkirkine. Thus far we have had insufficient quantities of the seco alkaloids to screen but are in the process of obtaining larger quantities.

In addition to the alkaloids, we have isolated from the methanol fraction, ethyl-1-hydroxy-4-oxo-2,5-cyclohexadiene acetate[c] (common name, jacaranone ethyl ester). The corresponding methyl ester jacaranone, isolated from Jacaranda caucana (Ogura, 1976) showed TC 165 against P388 lympoctic leukemia at a dose of 62 mg/Kg. Jacaranone[c] is one of the simplest naturally occurring antitumor agents. Jacaranone ethyl ester (NSC 289072) showed ED_{50} 3.3 x 10(0) in the KB screen and is presently undergoing in vivo screening.

As pointed out in our original grant application there is great danger in placing too much confidence in limited screening of plant extracts by NCI or any other screener. Thus, a number of examples exist where plant extracts showing negative screening results have yielded active compounds. However, in this case, with the experience we now have, we would proceed to isolate any new pyrrolizidine alkaloid available in a plant extract in reasonable amount and submit the pure alkaloid to NCI for screening, even if the plant extract showed little or no activity. This decision, of course, would be based on what our priorities were at a given time. Thus, obviously, an active plant extract would be of higher priority than an inactive one and a rational semi-synthetic alkaloid would be of highest priority.

Compounds Isolated and Identified 4/1/78 - 4/30/80

Table 2, Appendix B lists the thirty (30) novel compounds isolated and identified during the period indicated together with their structures. This list includes 13 pyrrolizidine alkaloids, 9 sesquiterpene lactones and furans, 4 monoterpenes and 4 miscellaneous aromatic compounds. Twenty two of these structures were determined using high resolution nuclear magnetic resonance spectroscopy and high resolution mass spectrometry. The following seven structures were determined by single crystal X-ray diffraction: the pyrrolizidine alkaloids retronecine, heliotrine and monocrotaline, the eremophilane sesquiterpene 3α-angeloyloxy-9-oxo-10αH-furanoeremophilane, the germacranolides 3β,8α-dihydroxy-6β,7α,11β H-germacran-4(15), 9(10)-dien-6, 12- olide and the corresponding 3-oxo-olide and the sesquiterpene 14-oxo-1,2-dihydrocacalol.methyl ether.

This is the first report of the isolation of these thirty compounds from these plant sources. However, all of the pyrrolizidine alkaloids except for the trans-cinnamic ester of hydroxysenkirkine have been reported from other plant sources. The following sesquiterpenes[c] are apparently new: 8α-ethoxy-10αH-eremophilenolide, 3α-angeloxyloxy-9-oxo-10αH-furanoeremophilane, 11βH-tatridin D (3β,8α-dihydroxy-6βH, 11βH-germacran-4(15), 9(10)-dien-6, 12-olide), 3-oxo-11βH-tatridin D, 11αH-gallicin, 11αH-1β-hydroxysant-4(14) en-6,12-olide C and this is the first report of δ-truxinic acid diethyl ester in a plant. Also, the monoterpenes filifolide·A and filifolide·B are quite unusual. Since we have been informed that NCI prefers to have 1.5 g of pure compound for in vivo screening, we have not pursued the screening of most of the above mentioned compounds, except for the pyrrolizidine alkaloids, since they do not occur in large amounts and the crude plant extracts did not show sufficient activity to warrant recollection of the plant material. Interestingly, europine N-oxide[a] and echinatine N-oxide[a] were found to be comparable in activity to indicine N-oxide against P388 lymphocytic leukemia tumors on direct comparisons under identical conditions (Zalkow, 1979). However, europine N-oxide and echinatine N-oxide are

c) The structure of this compound can be found in Table 2 of Appendix B.

derived from the base heliotridine while indicine N-oxide is derived from retronecine. These two bases differ in stereochemistry at C-7 (αOH in heliotridine, βOH in retronecine). In section D. Methods, we discuss the significance of these results, together with literature evaluations in the rationale for the semisynthetic work utilizing retronecine.

Critical Evaluation of NCI Screening Data of Pyrrolizidine Alkaloids and Derivatives[d]

Tables 3 and 4 of Appendix C show the pyrrolizidine alkaloids, their N-oxides and various derivatives that have previously been screened by NCI for activity against P388 lymphocytic leukemia tumors (PS). Among those that show significant activity (T/C) are: (-) and (+) 3,8-didehydroheliotridine (NSC 116336 and 144848), fulvine (NSC 089932), crispatine (NSC 089933), monocrotaline α-epoxide (NSC 113087), indicine N-oxide (NSC 132319), heliotrine N-oxide (NSC 030621), lasiocarpine N-oxide (NSC 035046), monocrotaline N-oxide (NSC 108378) and jaconine hydrochloride (NSC 030624). These data suggest that, in general, the N-oxides are more effective against the PS tumor system than the corresponding free amines.

The fact that indicine N-oxide has reached clinical evaluation is extremely significant.

To place this in proper perspective it should be noted that between 1965 and 1978 only 13 plant products or semisynthetic derivatives were filed by NCI with the FDA as candidates for clinical trials (indicine N-oxide being the most recent one), and of these, six have been since dropped because of uncontrolled toxicity, no therapeutic effect or insufficient activity (Suffness, 1979).

A surprising but apparently significant observation that arises from perusal of Tables 3 and 4 (Appendix C) is the effect of the vehicle used for administering the alkaloidal material. Not all of the alkaloidal materials were administered under the same conditions and some of the vehicles previously used have now been discontinued. Thus, some of the screening would have to be repeated to be meaningful.

Since indicine N-oxide has undergone the most extensive testing, it is possible to compare the effects of different vehicles on the efficacy of this drug. Seventy (70) experiments were carried out with indicine N-oxide in the PS tumor system employing intraperitoneal injections. Fifty one (51) of these used water, while nineteen (19) used normal saline as the vehicle. An average of the T/C values and optimum doses gave values of T/C 174 and 513 mg/Kg for water and T/C 120 and 726 mg/Kg for saline. However, not all of the testing was carried out under exactly the same conditions. It is clear that the optimum doses for water are considerably lower than those for saline. The data for indicine N-oxide thus shows that the vehicle water gives a higher survival rate at a significantly lower dose level. Finally, it can be seen that most of the pyrrolizidine alkaloids were not tested under these conditions and in the future we suggest water be used as the vehicle for the semisynthetic N-oxide analogs we submit for screening.

Semisynthetic Pyrrolizidine Alkaloid Analogs

In order to determine what features of the (-)trachelanthic acid side chain of indicine N-oxide were significant for antitumor activity, a semisynthetic program was initiated. Monocrotaline[a] was isolated from seeds of Crotalaria spectabilis Roth. obtained from South Georgia and hydrolysis of monocrotaline yielded the base retronecine, identical to that derived from indicine. The retronecine thus obtained was then acylated with various acids.

d) These data were kindly supplied by Dr. Matthew Suffness, Natural Products Branch, Developmental Therepeutics Program, Division of Cancer Treatment, National Cancer Institute, Silver Spring, Maryland 20910

In order to develop the synthetic techniques necessary to prepare the semisynthetic pyrrolizidine alkaloids we have thus far investigated the syntheses of twenty-one analogs (see Section D. Methods) in milligram quantities. We have spent approximately six months in developing the synthetic methods, isolation procedures and characterization methods and are now in a position to prepare these analogs in gram quantities for NCI screening. The details of our semisynthetic analog work can be found in Section D. Methods since it forms the basis of a major part of our future plans.

Publications and Unpublished Results

Publications and Official Presentations:

"Eremophilane Sesquiterpenes from Senecio aureus," Leon H. Zalkow, Leslie T. Gelbaum and Donald Van Derveer, J. Chem. Soc., Perkins Transactions I, 1542 (1979)[e].

"Pyrrolizidine Alkaloids from Middle Eastern Plants," L. H. Zalkow, S. Bonetti, L. Gelbaum, M. M. Gordon, B. B. Patil, A. Shani and D. Van Derveer, J. Natural Products, 42, 603 (1979)[e].

"A Phytochemical Investigation of Artemesia herba alba," L. H. Zalkow and M. M. Gordon, Proceedings of the 20th Annual Meeting of the American Society of Pharmacogonosy, July 29-Aug. 3, 1979, Purdue University, J. Natural Products, 42, 681 (1979).

"Pyrrolizidine Alkaloids from Senecio smallii," Leon Zalkow, Leslie Gelbaum and Sandra Bonetti, Proceedings of the 20th Annual Meeting of the American Society of Pharmacognosy, July 29-Aug. 3, 1979, Purdue University, J. Natural Products, 42, 688 (1979).

"Germacranolides from Artemisia herba alba," L. H. Zalkow, M. M. Gordon and D. Van Derveer, 178th ACS National Meeting, Washington, D. C., September 10-13, 1979.

"Secondary Plant Metabolites from Senecio smallii," Leslie Gelbaum and Leon H. Zalkow, 30th Southeastern Regional Meeting, American Chemical Society, Savannah, Ga., Nov. 8-10, 1978, Abstract 215.

Unpublished Material:

A perusal of Table 2 in Appendix B indicates that we have isolated and proven the structures of a number of compounds for which we have not yet published papers. We have completed publishable work on the following and manuscripts should be available soon:

1. "The Terpenes of Senecio smallii".
2. "Pyrrolizidine Alkaloids of Senecio smallii".
3. "The X-Ray Structures of Monocrotaline and Retronecine".
4. "A Phytochemical Investigation of Artemisia judaica".
5. "Germacranolides of Artemisia herba alba".

Changes in Project's Specific Aims from Previous Application

In the previous application we emphasized screening of plants, particularly from the genus Senecio in hopes of uncovering new leads. We have done this and, in addition, have received a number of other genera containing pyrrolizidine alkaloids and also potentially interesting plants which were physically accessible to the botanists (Artemisia, etc.) in their collections of the pyrrolizidine alkaloid bearing

e) Reprints of these papers are provided in Appendix D.

plants. After examining a rather large number of plants and having isolated quite a large number of novel natural products, our conclusion is that this procedure should, in the future, be secondary and our primary objective should now be to prepare semi-synthetic analogs. This, we believe and discuss in detail in the next section (D. Methods), will lead to a better understanding of the mechanism of action of indicine N-oxide and therefore to the ultimate goal - the discovery of better antitumor agents.

Also in the previous application we considered developing a structure activity relationship for eremophilane sesquiterpenoids, which also occur rather commonly in the Senecio. We do not now feel this should be a high priority goal since the species which contain these terpenes did not, in our hands, show sufficiently high activity to justify recollection. We have in hand a few such isolated compounds (see Appendix B, Table 2) but in insufficient amounts to get reliable in vivo screening data. We shall however request KB screening by NCI.

D. Methods: We will continue to submit plant extracts from the tribe Senecioneae and, in addition extracts from other pyrrolizidine bearing plants (Heliotropioideae and Boraginoideae) for screening by NCI. The plants will be collected and identified by botanists from Emory University, University of Georgia, Kansas State University, Hebrew University of Jerusalem, Valdosta State College, Virginia Polytechnic Institute and University, and Gordon Junior College who have performed this function for us in the past. The air-dried plant, after shipping to the Natural Products Laboratory at the School of Chemistry, Georgia Institute of Technology will be ground and extracted. We are well equipped for these operations, having all size Soxhlet extractors including three of five kilogram capacity.

Recently NCI has revised its recommended extraction procedure (5/24/78) for extracts to be supplied to them. This new procedure requires extraction of the dried plant material with 95% ethanol followed by partitioning of the residue obtained on removal of the ethanol between chloroform and water. They then recommend only screening the chloroform fraction. While this procedure may be of general use, it is clearly a mistake for pyrrolizidine alkaloid-bearing plants, since almost all of them, if not all, contain water soluble N-oxides (Bull, 1968), which as previously mentioned are of more importance than the free bases in regard to antitumor activity. Therefore, we will process the water fraction for N-oxides and, of course, screen the isolated alkaloids.

Because, as previously mentioned, it is not uncommon in this area, that inactive crude plant extracts yield active alkaloids (this appears to be due to a concentration effect, the alkaloids being present in low concentration and being active at high concentration), we plan to screen the crude 95% ethanol extracts, chloroform and water partitions, and the isolated alkaloid fractions in each case.

Isolation of the alkaloid fractions will be conducted as follows. The free bases will be isolated from the chloroform partition residue by extraction with 2N sulfuric acid followed by basification of the sulfuric acid extract with ammonium hydroxide, and reextraction back into chloroform. Evaporation of the chloroform will yield the crude alkaloid fraction which will be screened. If this fraction shows activity it will be separated by high performance liquid chromatography into individual alkaloids, each of which will be screened. If the crude alkaloid fraction is found to be inactive, no further separation of this material will be undertaken.

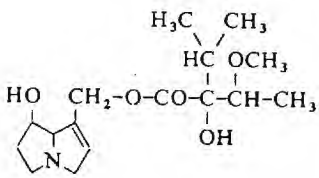
The previously mentioned water partition, which contains the pyrrolizidine alkaloid N-oxides, will be reduced with zinc and sulfuric acid (Bull, 1968), then the solution will be basified with ammonium hydroxide and extracted with chloroform to give the free bases which will be processed as above. The free bases will then be converted

into their corresponding N-oxides with hydrogen peroxide (Bull, 1968) or microbiologically (see below). This will insure that we do not miss any unusual alkaloids in the water fraction during the above separation procedure.

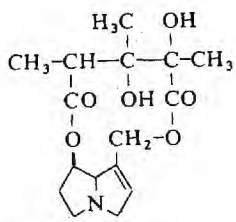
Separations of crude alkaloid fractions will be done by high performance liquid chromatography. We have become very proficient at separating these materials using a combination of silica gel and reverse phase HPLC columns. We are able to process twenty (20) g of crude alkaloid at one time through our Waters 500 Preparative HPLC instrument and then we are able to prepare analytically pure samples in gram quantities by further high pressure HPLC using both ultraviolet and refractive index detectors. Since we also have developed the capability to prepare our own HPLC columns (reverse phases C-18, C-8, C-2; silver nitrate on silica) we are convinced we can separate any natural alkaloid fraction into its components.

Once the alkaloids have been isolated in pure form, their characterizations will be done using the usual techniques, but in particular, high resolution ^{1}H and ^{13}C nuclear magnetic resonance spectrometry (100 and 300 mHz) and high resolution mass spectrometry. We have already gained a great deal of experience using these techniques with numerous pyrrolizidine alkaloids (Zalkow, 1979) and are confident that we can now pick up small variations in the necic acid side chains of the alkaloids by careful examination of the high resolution NMR spectra.

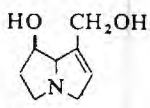
In the School of Chemistry at the Georgia Institute of Technology single crystal X-ray diffraction is a routine service and thus we will get X-ray structures of all suitable crystalline alkaloids. We have already done this for heliotrine, monocrotaline and the base retronecine. Computer drawn pictures of these three substances are indicated below (the drawing of monocrotaline is the mirror image of the natural material). We plan to use the information from X-ray analyses to try and determine the structural and conformational features that make indicine N-oxide such a good antitumor agent as compared to, for example, heliotrine N-oxide.. In order to do this we need to obtain the X-ray structure of indicine and compare it to that of heliotrine and then we need to compare the X-ray structures of the N-oxides to each other and to their respective bases. Thus an examination of the computer drawn picture of heliotrine clearly indicates the endo puckering of the pyrrolizidine ring and the absence of intramolecular hydrogen bonding between the methoxy oxygen (05) and the OH group (01) at C-7. What is the situation in indicine, which differs from heliotrine (see structures below) in stereochemistry at the C-7 hydroxyl group and in having a free hydroyl group at C-12 instead of a methoxyl? Thus, is indicine more like monocrotaline? How much effect does the N-oxide have on conformation? Of course all of these arguments will be based on crystal structures and may not apply to these molecules in solution. However, we may be able to say something about the relative ease with which indicine and heliotrine form macrocyclic hydrogen bonds.



Heliotrine
(heliotridine, heliotric acid)



Monocrotaline
(retronecine, monocrotalic acid)



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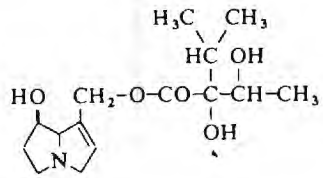
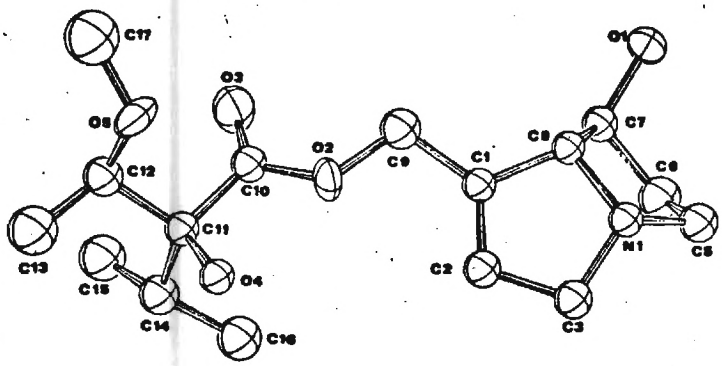
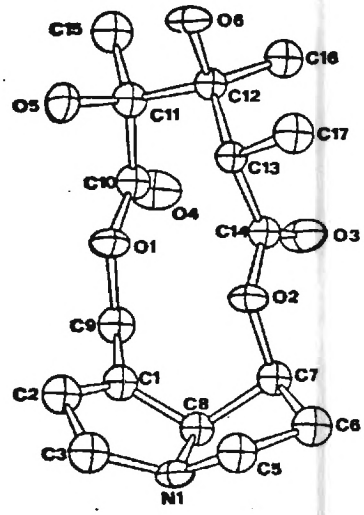


Indicine
(retronecine,
(−)-trachelanthic acid)

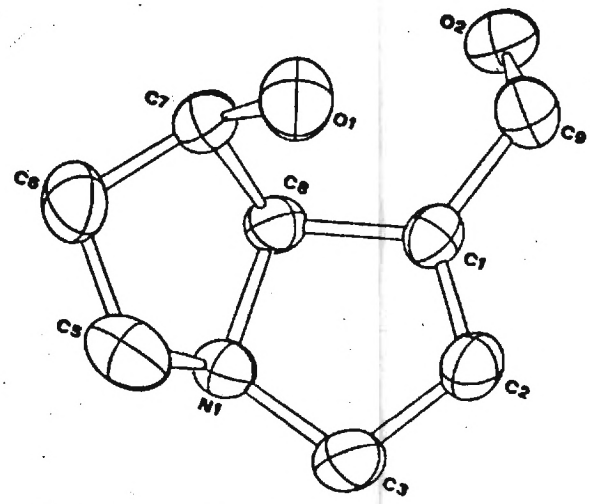
See next page for computer drawn pictures.



HELIOTRINE



MONOCROTALINE



RETRONECINE

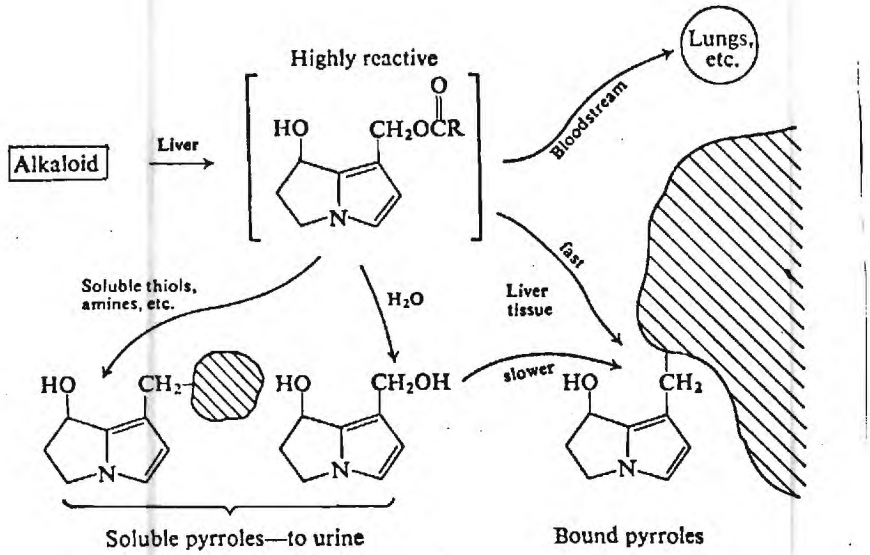
Based on our experience during the first two years of this grant, we conclude that the most rapid method of discovering new antitumor agents belonging to the pyrrolizidine alkaloid class will be by semisynthetic methods. Our reasoning is based on the following observations: (1) screening a large number of plants seldom yields new and novel pyrrolizidine alkaloids. However, the screening is still worthwhile in the long run because when a new alkaloid is found, it will usually be complicated enough as to not be synthetically accessible and will provide a model for semisynthetic compounds. (2) The state of the art in synthetic organic chemistry is such that we cannot expect totally synthetic pyrrolizidine alkaloids in the near future. A number of the most distinguished organic chemists in America have been working for several years on the syntheses of the bases retronecine (see p.) and its C-7 epimer, heliotridine, and apparently the most we can expect from these efforts, at this time, will be academic syntheses without the provision of any reasonable quantitites of these materials. (3) Retronecine and its N-oxide is available to us in hundreds of gram quantities as by-products from the isolation of indicine N-oxide from Heliotropium indicium (see letters in Appendix E). (4) Preliminary studies in our laboratory (see C. Progress Report) and by others (Atal, 1978; Hoskins, 1977; Culvenor, 1976) have shown that we can make gram quantities, enough for in vivo screening by NCI, of a large number of pyrrolizidine alkaloids by acylation of retronecine derived from natural sources.

Which semisynthetic compounds should be prepared? The goal is obviously to separate the toxic effects of pyrrolizidine alkaloids from the antitumor activity. This, of course, is easily said but the solution to this is one of the major problems in cancer research. There has been extensive work on the toxic action of pyrrolizidine alkaloids (McLean, 1970; Culvenor, 1976; Schoental, 1979) and the structural features of the alkaloids which enhance toxicity are known, but the biochemical basis of the toxicity is still not very well understood. Almost nothing is known of the mechanism of the antitumor activity (Powis, 1979).

Diesters of heliotridine and retronecine are about 4 times as toxic as the respective mono-esters and heliotridine esters are 2-4 times as toxic as retronecine esters (Culvenor, 1976). Thus, fortunately, the readily available base retronecine is the base of choice and chemically the C-9 monoester is readily prepared because of the greater reactivity of the C-9 allylic hydroxyl group (Hoskins, 1977). Therefore, monoesters of retronecine, esterified at C-9, which are analogs of indicine should be readily available. N-oxides are substantially less toxic than their corresponding free bases (Culvenor, 1976) when administered by the intraperitoneal route, but not when given orally since they are quickly reduced to the alkaloid in the gastro-intestinal tract, because of their decreased lipid solubility. The synthetic analogs, just as the natural alkaloids, can be readily oxidized to the N-oxides with hydrogen peroxide (Bull, 1968).

The toxicity, and perhaps the antitumor activity, of the pyrrolizidine alkaloids are apparently due to metabolites of the pyrrole type formed by microsomal mixed function oxidases (Mattocks, 1972). It has been suggested that these pyrroles act as alkylating agents as illustrated in the diagram (Mattocks, 1972).

The interst in pyrrolizidine alkaloids as antitumor agents apparently arose from observations of the long-lasting effect of single doses, possibly related to cell division (McLean, 1970). Liver cells suffering the long term effects of pyrrolizidine alkaloid poisoning accumulate an excess of nuclear chromatin and fail to divide (Downing, 1968). The divorce of DNA synthesis from cell division is extremely significant. Pyrrolizidine alkaloids are capable of causing chromosome breakage in the growing cells of the root tip of the onion and of wild peas (Avanzi, 1962). Cysteine protects the plant tissues against this action. The alkaloids cause a significant

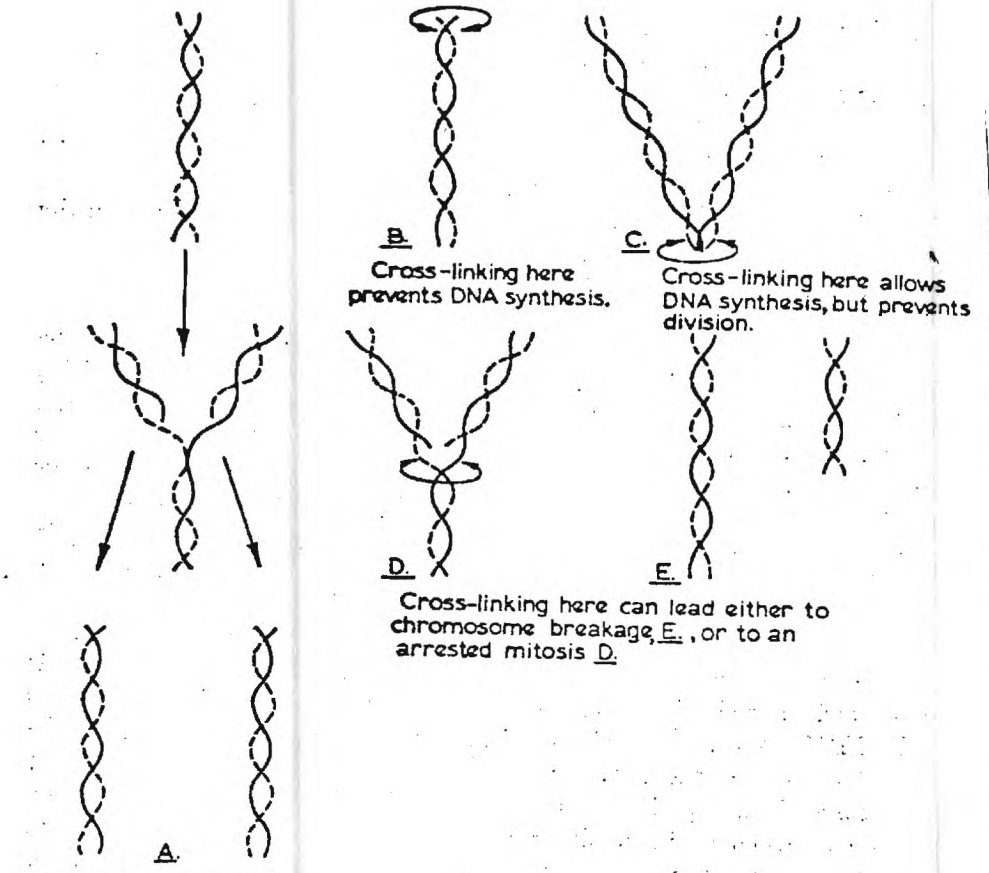


Hypothetical fate of a reactive pyrrolic metabolite (dihydropyrrolizine ester) in the liver of a rat

number of mutants in successive broods of Drosophilia and the effect is dose-dependent (Clark, 1960; Cook, 1966). Pyrrolizidine pyrroles have mutagenic activity in Drosophilia and Aspergillus and are capable of inhibiting the DNA virus, infectious bovine rhinobracheitis (McLean, 1970). It has been speculated that pyrrolizidine pyrroles act by cross linking DNA, but experimental evidence on this point is scant. Culvenor et al. (Culvenor, 1969) showed that labelled alkaloid transferred its label to rat nucleic acids in amounts roughly equivalent to one molecule of alkaloid per molecule of DNA. On the next page is a diagram of how these pyrroles might interfere with DNA replication (McLean, 1970). We have no indication of how the cross-linking actually occurs - perhaps via the C-7 hydroxyl group or the cross-linking may involve one covalent bond and one hydrogen bond.

The above discussion illustrates the problem with most antitumor agents - their lack of specificity. We would like, ultimately, to design an antitumor agent in inactive or latentiated form which is activated by an enzyme associated with the targeted tumor tissue (Marguisee, 1978). We discuss our plans along this line later in the proposal. Meanwhile, we will describe more immediate, short range work which should provide useful structure-activity information.

The necic acid side chains of the pyrrolizidine alkaloids are apparently not metabolized into active compounds. Their role in toxicity, antimitotic and anti-tumor activity is not understood. However, their role is obviously critical as seen by the fact that indicine N-oxide is less toxic and a better antitumor agent than closely related alkaloids with the same necine base (retronecine) and slightly different necic acids. Mattocks (Mattocks, 1973), one of the leading authorities in the world on pyrrolizidine alkaloids has clearly stated the case for preparing semisynthetic analogs as proposed here - "Thus it is evident that by varying the acid moiety the tissue distribution and toxic effects of pyrrolic metabolites can be altered. This has relevance to the design of possible cytotoxic and antimitotic derivaties for antitumor therapy."



A, normal replication of DNA and separation of chromatids; B, cross linking of DNA here prevents replication; C, cross-linking of DNA here allows replication but prevents separation of chromatids; D, cross linking here allows partial DNA replication and leads to arrest of mitosis or to chromosome breakage, E.

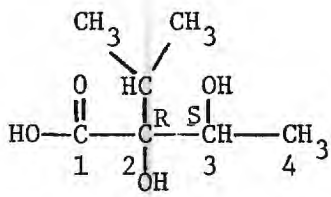
Table 1 lists 21 semisynthetic analogs which we have recently prepared on a small scale in order to develop our synthetic procedures, isolation techniques and characterizations. The selection of compounds was designed to give an initial fairly broad spectrum of compounds so that the screening data would be instructive in terms of structure-activity relationships. These analogs were prepared by coupling retronecine with the appropriate acid using 1,1'-carbonyldiimidazol (CDI) (Hoskin, 1977) as the coupling agent. None of the compounds in Table 1, except for one have been previously reported, and none have been screened for antitumor activity. The synthetic procedure can, in principal, yield three products in each case, the C-7 and C-9 monoesters and the 7,9-diester. In fact, the reactivity of the C-9 hydroxyl group is so much greater than the C-7 hydroxyl group, that in almost every case the C-9 isomer, the desired isomer, predominates to such an extent that we only isolate it. Two exceptions can be seen in Table 1. Thus, benzoic acid yielded the C-9 monoester 6 and the diester 7 and phenylacetic acid gave the C-9 ester 8 and C-7 ester 9.

Compounds 1 through 16 of Table 1 were readily purified by HPLC using activity III alumina and they have been characterized by high resolution FT NMR analyses and high resolution mass spectral analyses using chemical ionization and exact mass determination. Our next goal is to prepare these compounds in gram quantities for screening by NCI (see Appendix E). Where possible, the analogs will be converted into their N-oxides which will also be screened.

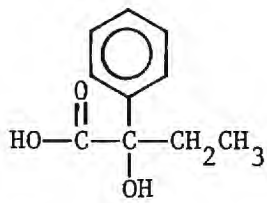
Compounds 1-5 and their N-oxides will provide leads on the effect of lipophilicity, steric bulk near the acyl carbonyl and the effect of a conjugated double bond. The N-oxides of 1,2, 4 and 5 should present no problem in preparation by the usual method with hydrogen peroxide. However, this method when used with 3 would be expected to yield the epoxide N-oxide, which would be an interesting compound for screening in its own right. In conjunction with Professor S. Crow a microbiologist from Georgia State University, we have found what appears to be a good organism for specifically converting pyrrolizidine alkaloids to N-oxides. Thus, we expect to explore this microbiological oxidation further with the goal of using it to prepare N-oxides of those compounds which have other groups sensitive to oxidation with hydrogen peroxide.

Compounds 6-14 all contain one or more phenyl rings in the acyl moiety. A comparison of the screening data of 8 and 9 will measure the relative effects of acylation at C-9 and C-7 respectively, while a comparison of the data from 6 and 7 will give an indication of the effect of mono vs diacylation. A comparison of the data from 6, 8 and 10 will give information on the effect of moving the phenyl group further away from the carbonyl group. Compound 11 will test the effect of a second phenyl group, 12 will measure the effect of a conjugated phenyl group (compared to 10) and 13 will measure the effect of the strong electron withdrawing chlorine atom in the ortho position (compared to 6). Compound 15 will measure the effect of a pyridine ring vs a phenyl ring (compare with 6).

Compound 14 is particularly interesting as seen when its side chain, 2-phenyl-2-hydroxybutyric acid, is compared with the (-) trachelanthic acid side chain of indicine. Thus, the phenyl ring replaces the isopropyl group at C-2 and the C-3



(-) trachelanthic acid

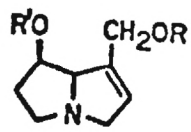


(±) 2-phenyl-3-hydroxybutynic acid

hydroxyl group is missing. (±) 2-Phenyl-2-hydroxybutyric acid is readily prepared by the addition of HCN to propiophenone followed by acid hydrolysis of the cyanohydrin. We plan to prepare a number of analogs related to 14 in which the acyl side chain most closely resembles that in indicine. Compound 14 was prepared as the first model in this series because the above described reaction is so facile in this case. We, of course, do not know at this time whether or not the chirality of the side chain is very important.

Compound 16 was prepared inadvertently as a by-product in the preparation of 3 and apparently arises from the reaction of 3 with imidazole, from the CDI coupling reagent, or prior reaction of β-methyl-crotonic acid with CDI to give the Michael adduct. It's unusual structure appears to be of interest to NCI (see Index E). Compound 17 deserves special note. We had previously prepared the acid, tetrahydro-4-methylene-5-oxo-3-furoic acid (NSC 124901) which has been of great interest to NCI since it has shown good activity in the PS system (T/C 148, 32 mg/Kg). We have just sent NCI an additional 25 g of this acid for more extensive screening. We therefore decided to couple this acid with retronecine. At present this product still requires further purification but 17 is a particularly exciting prospect.

TABLE 1



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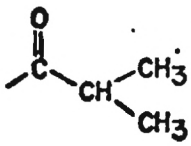
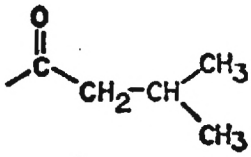
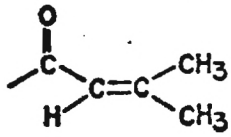
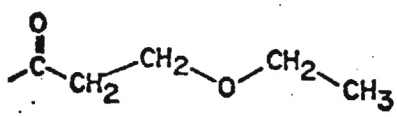
1.		H
2.		H
3.		H
4.		H
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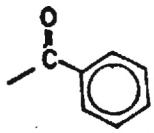
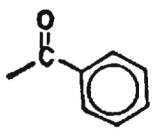
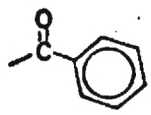
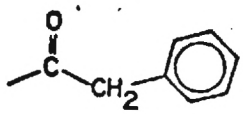
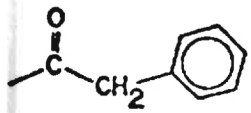
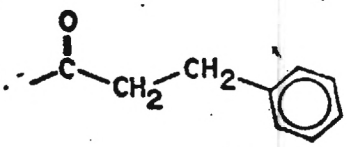
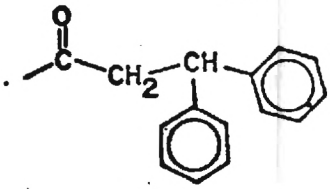
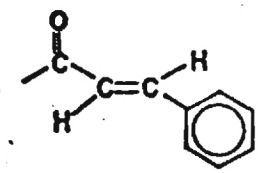
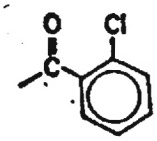
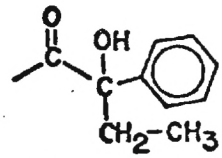
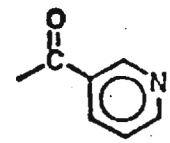
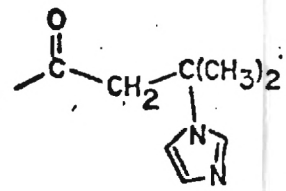
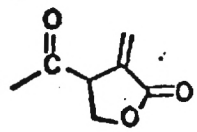
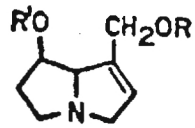
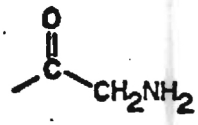
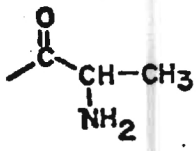
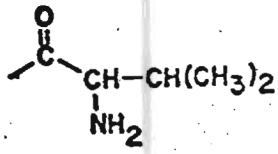
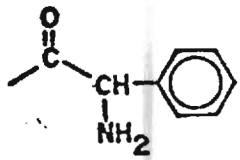
6.		H
7.		
8.		H
9.	H	
10.		H
11.		H
12.		H
13.		H
14.		H
15.		H
16.		H
17.		H

TABLE 1 Continued



	R	R'
18.		H
19.		H
20.		H
21.		H

As long ago as 1953, latentiated nitrogen mustards were prepared (Danielli, 1954). At that time, these latentiated antitumor agents could not exploit the differences between normal and tumor tissue. Recently, workers (Marguisee, 1978) have attempted to exploit tumor-associated collagenase by using a nitrogen mustard inactivated by a collagenase-sensitive peptide. Collagenase is a highly specific collagen-degrading enzyme which is elaborated by many, if not all, slow-growing tumors. Tumor-associated collagenase cleaves the peptidyl acyl moiety Cbz-L-Pro-L-Leu-Glz-L-Pro-Glz (Wünsch, 1963). Thus, we ultimately plan to attach this acyl peptidyl group to retronecine. However, at this stage we are beginning by attaching single amino acids to the C-9 position of retronecine and compounds 18-20 of Table 1 illustrate our initial efforts. Compounds 18-21 were prepared by coupling the N-t-butoxycarbonyl (t-BOC) derivatives of the appropriate amino acids with retronecine in the presence of CDI. The t-BOC group is later removed by reaction with dry HCl in dioxane. We next plan to prepare di and tripeptide derivatives and finally the target pentapeptide mentioned above. This pentapeptide will be prepared as previously described (Marguisee, 1978) and coupled to retronecine.

Finally we plan to prepare dihydropyrrolizine derivatives (pyrroles) of the semisynthetic pyrrolizidine alkaloids according to literature procedures (Culvenor, 1969). As previously mentioned, the dihydropyrrolizidine (pyrrole) compounds are known metabolic products of pyrrolizidine alkaloids, but we do not know if they are the active antitumor agents or if metabolism must occur <u>in vivo</u> to produce the desired activity.